Research paper

New steroidal 17β-carboxy derivatives present anti-5α-reductase activity and anti-proliferative effects in a human androgen-responsive prostate cancer cell line

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A B S T R A C T

The androgens testosterone (T) and dihydrotestosterone (DHT), besides playing an important role in prostate development and growth, are also responsible for the development and progression of benign prostate hyperplasia (BPH) and prostate cancer. Therefore, the actions of these hormones can be antagonized by preventing the irreversible conversion of T into DHT by inhibiting 5α-reductase (5α-R). This has been a useful therapeutic approach for the referred diseases and can be achieved by using 5α-reductase inhibitors (RIs). Steroidal RIs, finasteride and dutasteride, are used in clinic for BPH treatment and were also proposed for chemoprevention of prostate cancer. Nevertheless, due to the increase in bone and muscle loss, impotency and occurrence of high-grade prostate tumours, it is important to seek for other potent and specific molecules with lower side effects. In the present work, we designed and synthesized steroids with the 3-keto-17β-fluoro-1α-carboxyl moiety in the A-ring, as in the 5α-R substrate T, and with carboxamide, carboxyester or carboxylic acid functions at the C-17 position. The inhibitory 5α-R activity, in human prostate microsomes, as well as the anti-proliferative effects of the most potent compounds, in a human prostate microsomes, as well as the anti-proliferative effects of the most potent compounds, in a human androgen-responsive prostate cancer cell line (LNCaP cells), were investigated. Our results showed that steroids 3, 4 and 5 are good RIs, which suggest that C-17β lipophilic amides favour 5α-R inhibition. Moreover, these steroids induce a decrease in cell viability of stimulated LNCaP cells, in a 5α-R dependent-manner, similarly to Finasteride.

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Abbreviations: AR, androgen receptor; ATCC, American Type Culture Collection; BOP, (benzotriazol-1-yl oxy)tris(dimethylamino)phosphonium hexafluorophosphate; BPH, benign prostate hyperplasia; BSA, bovine serum albumin; CFBS, pre-treated charcoal heat-inactivated fetal bovine serum; DHT, dihydrotestosterone; DHT-13C4, dihydrotestosterone-13C4; DMSO, dimethyl sulfoxide; DTE, 1,4-dithioerythritol; DTT, dithiothreitol; DHT-13C3, dihydrotestosterone-13C3; E13C17, 17α-[13C(1)]-N-(trimethylsilyl)trifluoroacetamide; FBS, fetal bovine serum; FDA, Food and Drug Administration; GC—MS, gas chromatography—mass spectrometry; LDH, lactate dehydrogenase; F, finasteride; DLLME, dispersive liquid–liquid microextraction; LNCaP cells, human androgen-responsive prostate cancer cell line; MeCN, acetonitrile; MeOH, methanol; MSTFA, N-methyl-N-(trimethylsilyl)trifluoroacetamide; MTIT, tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; NADPH, nicotinamide adenine dinucleotide phosphate; 5α-R, 5α-reductase; RIs, 5α-reductase inhibitors; RPMI, Roswell Park Memorial Institute; RT, room temperature; SEM, standard error of mean; T, testosterone; T-d3, testosterone-d3 solution.

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1. Introduction

The androgens testosterone (T) and dihydrotestosterone (DHT) (Fig. 1) play an important role not only in normal prostate development, cell proliferation and growth, but also in the genesis and progression of benign prostate hyperplasia (BPH) and prostate cancer [1–3]. BPH is a recurrent disease in 50% of males by the age of 50 and 90% of males by the age of 80, being the major responsible for men morbidity [4], while prostate cancer is the second-leading cause of cancer death in men [1]. T is the most abundant circulating androgen and it is converted in prostate into DHT by the enzyme 3-oxo-5α-steroid-4-dehydrogenase. 5α-reductase (5α-R) (Fig. 1). DHT is the main androgen in the prostate and the major responsible for differentiation and prostate growth [4,5].

As androgens are implicated in the development and progression of BPH and prostate cancer, the treatment of these diseases can be achieved by antagonizing the action of these hormones by preventing the irreversible conversion of T into DHT, through the inhibition of 5α-R [3,4,6]. The 5α-R inhibitors (RIs) can be classified into steroidal and non-steroidal [5]. The development of non-steroidal RIs has increased in the last years, but they did not show promising inhibitory activities of 5α-R when compared to steroidal compounds. The first steroidal RIs were designed by modifications of the T, the natural substrate of the enzyme. One of the main modifications was the substitution of one carbon atom of the A-, B-, C- or D-rings of the steroid framework by a heteroatom, specially nitrogen, leading to the discovery of potent inhibitors of human 5α-R such as 4-azasteroids, 6-azasteroids and 10-azasteroids [7,8]. The 4-azasteroid finasteride (Fig. 2) was the first RI to be clinically approved for the treatment of BPH in 1992 [9,10]. Ten years later, another 4-azasteroid, dutasteride (Fig. 2), was also approved by FDA to be used for the symptomatic treatment of BPH [6]. Finasteride and dutasteride are potent irreversible inhibitors of 5α-R forming strong ternary complexes with 5α-R-NADPH complex [5,11]. Both RIs reduce the intraprostatic DHT levels and, therefore, the prostatic size [3,12], being currently used in the clinic for the treatment of BPH and were also proposed for chemoprevention and treatment of prostate cancer [13,14]. Despite the success of finasteride and dutasteride they still have some disadvantages like the increase in bone and muscle loss, and impotency. Furthermore, different clinical trials have demonstrated that these RIs increased the risk of high-grade prostate cancer, which prevent FDA approval of finasteride and dutasteride to be used for prostate cancer treatment [5,15]. For this reason, it is important to seek for other potent and specific molecules with lower side effects.

Previous studies highlight the importance of the 3-keto-Δ4- androstane-17β-carboxamide steroids as 5α-R inhibitors [16–18]. It has also been found that substitution at C-17β position of steroids by lipophilic side chains, containing amide or ester groups, enhances 5α-R inhibitory activity by binding to a lipophilic pocket on the enzyme [6]. In the present work, it was designed and synthesized steroids with the 3-keto-Δ4 moiety in the A-ring, as in T, and with carboxamide, carboxyster or carboxylic acid functions at the C-17β position (Scheme 1). The rationale particularly focus on the carboxamide analogues of finasteride and dutasteride (Fig. 2).

obtained by combining in the same molecule, the A-ring of the substrate T with the C-17β carboxamide group of the referred RIs. In addition, the biochemical activity of the synthesized compounds was determined in human prostate microsomes, by a new methodology recently developed by our group [19]. The antiproliferative effects of the most potent RIs and of finasteride were also investigated in a human androgen-responsive prostate cancer cell line (LNCaP cells).

2. Material and methods

2.1. Chemistry

Reactions were controlled by TLC using silica gel 60 F254 plates. Melting points (MPs) were determined on a Reichert Thermopan hot block apparatus and were not corrected. IR spectra were recorded on a Jasco 420 FT/IR spectrometer. The 1H NMR spectra were recorded at 600 MHz, on a Varian Unity 600. The 13C NMR spectra were recorded at 150 MHz on a Varian Unity 600. Chemical shifts were referred in δ values in parts per million (ppm) downfield from tetramethylsilane as an internal standard. All J values are given in Hz. Mass spectra ESI were obtained with a mass spectrometer QIT-MS Thermo Finningan, model LCQ Advantage MAX. Purity was determined by HPLC using a Liquid Chromatograph of High Performance Thermo Finningan. 4-Androstene-3-one-17β-carboxylic acid (1) was purchased from Fountain Limited (Malta). Reagents and solvents were used as obtained from suppliers without further purification, with exception of dichloromethane, and pyridine, which were dried through reflux and distillation from CaH2, being stored away from light in a brown bottle with type 4 A molecular sieves, under an atmosphere of dry N2.

All compounds possess a purity superior to 98%. The purity was checked by HPLC with a C18-reversed phase column and water/ acetonitrile 30:70 as solvent. The purity of individual compounds was determined from the area peaks in the chromatogram of the sample solution.

2.1.1. 4-Androstene-17β-carboxylic acid (2)

Sodium borohydride (590.3 mg, 15.6 mmol) was added in small portions to a cooled and stirred mixture of trifluoroacetic acid (3.6 mL), glacial acetic acid (3.6 mL) and acetonitrile (3.6 mL). After this, a solution of compound 1 (1.0 g, 3.16 mmol) in dry dichloromethane (33 mL) was added and the reaction proceeded at room temperature, under a stream of dry nitrogen, until all the starting material had been consumed (3 h 20 min by TLC). The reaction mixture was neutralized with 10% NaHCO3 extracted with chloroform (4 × 100 mL) and the organic layer washed with water (4 × 100 mL), dried over anhydrous Na2SO4, filtered and concentrated to dryness. Crystallization from methyl acetate/hexane afforded the pure compound 2 (680.5 mg, 71%) as white crystals. Mp(ethyl acetate/hexane): 205–208 °C; IR (NaCl plates, CHCl3); vmax = 3399 (O–H carboxylic acid), 3020 (H–C=), 1701 (C=O carboxylic acid), 1676
2.1.2. General method for obtaining N-tert-butyl-3-oxoandrost-4-en-17β-carboxylic acid (5)

Compound 1 was dissolved in N,N-dimethylformamide and triethylamine. The solution was cooled in an ice-water bath and the amine was added followed by a solution of (benzotriazol-1-yl)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) in dichloromethane. The reaction mixture was stirred at 0°C for 30 min and then at room temperature for 1 h 20 min to 3 h. Dichloromethane was removed under reduced pressure and the resulting solution diluted with water (25 mL) and extracted with ethyl acetate (2 × 25 mL). The organic layer was washed successively with HCl 1 N (25 mL), water (25 mL), NaHCO3 1 M (3 × 25 mL) and water (2 × 25 mL), dried over anhydrous MgSO4, filtered and concentrated to dryness. The residue obtained was purified by silica gel column chromatography with petroleum ether 40–60 °C/ethyl acetate gradient, except for compound 7, giving the pure compounds.

2.1.2.1. N-tert-Butyl-3-oxoandrost-4-en-17β-carboxamide (3)

Compound 1 (200.0 mg, 0.632 mmol); N,N-dimethylformamide (1.3 mL); triethylamine (0.1 mL, 0.72 mmol); propylamine (0.1 mL, 0.95 mmol); BOP (279.1 mg, 0.63 mmol); dichloromethane (1.6 mL); reaction time: 1 h 20 min; yield (122.6 mg, 54%). Mp (petroleum ether 40–60 °C); IR (NaCl plates, CHCl3): \( \nu_{\text{max}} = 3368 \) (N–H), 1656 (C=O amide and C=O ketone), 1617 (C=C) cm\(^{-1}\); \(^1\)H NMR (CDCl3, 600 MHz): \( \delta = 0.72 \) (3H, s, 18-H), 0.92 (3H, t, \( J = 7.0 \) Hz, -CH3), 1.18 (3H, s, 19-H), 2.40 (1H, dd, \( J = 4.9 \), 17a-H), 3.03–3.12 (2H, m, CH2), 5.28 (1H, bs, –NH), 5.73 (1H, s, 4-H); \(^13\)C NMR (CDCl3, 150 MHz, CDCl3): \( \delta = 13.1 \) (C-18), 17.4 (C-19), 21.0, 23.2, 24.9, 29.0 (C3), 31.9, 32.8, 34.0, 35.6, 37.5, 38.5, 38.6, 43.5, 51.1, 53.8, 55.6, 57.5, 123.9 (C-4), 171.1 (C-5), 171.6 (C20=O), 199.5 (C3=O); ESI m/z 372.6 ([M + H]+, 100%).

2.1.2.2. N-Propyl-3-oxoandrost-4-en-17β-carboxamide (4)

Compound 1 (200.0 mg, 0.632 mmol); N,N-dimethylformamide (1.3 mL); triethylamine (0.1 mL, 0.72 mmol); propylamine (0.1 mL, 0.95 mmol); BOP (279.1 mg, 0.63 mmol); dichloromethane (1.6 mL); reaction time: 1 h 20 min; yield (122.6 mg, 54%). Mp (petroleum ether 40–60 °C/ethyl acetate): \( \nu_{\text{max}} = 149–147 \) °C; IR (NaCl plates, CHCl3): \( \nu_{\text{max}} = 3368 \) (N–H), 1656 (C=O amide and C=O ketone), 1617 (C=C) cm\(^{-1}\); \(^1\)H NMR (CDCl3, 600 MHz): \( \delta = 0.72 \) (3H, s, 18-H), 0.92 (3H, t, \( J = 7.0 \) Hz, -CH3), 1.18 (3H, s, 19-H), 2.40 (1H, dd, \( J = 4.9 \), 17a-H), 3.03–3.12 (2H, m, CH2), 5.28 (1H, bs, –NH), 5.73 (1H, s, 4-H); \(^13\)C NMR (CDCl3, 150 MHz, CDCl3): \( \delta = 11.4 \) (C-18), 17.3 (C-19), 20.9, 23.1, 23.6, 24.4, 31.9, 32.8, 33.9, 35.6, 35.7, 38.6, 41.2, 43.6, 53.8, 55.5, 57.0, 123.8 (C-4), 171.1 (C-5), 172.4 (C20=O), 199.5 (C3=O); ESI m/z 358.8 ([M + H]+, 100%).

2.1.2.3. N-Hexyl-3-oxoandrost-4-en-17β-carboxamide (5)

Compound 1 (177.8 mg, 0.562 mmol); N,N-dimethylformamide (1.3 mL); triethylamine (0.09 mL, 0.64 mmol); hexylamine (0.11 mL, 0.84 mmol); BOP (167.2 mg, 0.38 mmol); dichloromethane (1.6 mL); reaction time 3 h; yield (78.4 mg, 35%). Mp (petroleum ether 40–60 °C/ethyl acetate): 56–61 °C; IR (NaCl plates, CHCl3): \( \nu_{\text{max}} = 3389 \) (N–H), 1656 (C=O amide and C=O ketone), 1621 (C=C) cm\(^{-1}\); \(^1\)H NMR (CDCl3, 600 MHz): \( \delta = 0.72 \) (3H, s, 18-H), 0.87 (3H, t, \( J = 7.0 \) Hz, -CH3), 1.18 (3H, s, 19-H), 2.40 (1H, dd, \( J = 4.9 \), 17a-H), 3.03–3.25 (2H, m, CH2), 5.28 (1H, bs, –NH), 5.73 (1H, s, 4-H); \(^13\)C NMR (CDCl3, 150 MHz, CDCl3): \( \delta = 13.2 \) (C-18), 17.3 (C-19), 20.9, 23.1, 23.6, 24.4, 31.9, 32.8, 33.9, 35.6, 35.7, 38.6, 41.2, 43.6, 53.8, 55.5, 57.0, 123.8 (C-4), 171.1 (C-5), 172.4 (C20=O), 199.5 (C3=O); ESI m/z 358.8 ([M + H]+, 100%).
2.2.1. Stock solutions

The individual stock internally standard solutions of testosterone-\(d_3\) (T-\(d_3\); 98 atom%) D (Fluka, Neu-Ulm, Germany) at 3.47 \(\mu\)M and dihydrotestosterone \(^{13}C_2\) (DHT-\(^{13}C_2\); 99 atom%) \(^{13}C\) (Sigma–Aldrich, Chemie GmbH, Steinheim, Germany) at 3.44 \(\mu\)M were prepared in MeCN (Fluka). The stock solution of each steroid was prepared in 100% DMSO (Sigma–Aldrich). The stock solution of testosterone (T, 99% purity grade) (Fluka) and dihydrotestosterone (DHT, 99% purity grade) (Sigma–Aldrich) was prepared in MeOH for gas chromatography–mass spectrometry (GC–MS) analysis (Fluka) or prepared in absolute ethanol (Fluka) for cell culture. All these solutions were stored at \(-20^\circ\) C before use. Appropriate dilutions were freshly prepared just prior to the assays and the final concentration of DMSO and ethanol in culture medium was less than 0.05% and 0.01%, respectively.

2.2.2. Preparation of prostate microsomes

Human prostate microsomes were prepared as previously described [19] and according to [21–23], with some modifications. Human prostate tissues, from BPH patients, provided by a local hospital after patient informed consent, were collected after surgery and placed in cold 20 mM sodium phosphate buffer (pH 6.5), washed and stored at \(-8^\circ\) C before use. Briefly, to prepare prostate microsomes, the prostatic tissues were weighed and homogenized in a Polytron homogenizer with a solution of 20 mM sodium phosphate buffer (pH 6.5) containing 0.32 M sucrose and 0.1 mM dithiothreitol (DTT; 1:1, w/v) (Sigma–Aldrich). The homogenate was centrifuged at 5000 \(\times\) g for 30 min. The supernatant obtained was centrifuged at 20,000 \(\times\) g for 30 min and then at 54,000 \(\times\) g for 45 min to obtain the microsomal pellet, that was washed and resuspended in 20 mM sodium phosphate buffer (pH 6.5) containing 0.32 M sucrose, 0.1 mM DTT and glycerol and stored at \(-80^\circ\) C. The protein content was determined by the Bio-Rad protein assay (Bio-Rad, Laboratories Melville, NY, USA) using bovine serum albumin (BSA) (Sigma–Aldrich) as standard. All the procedures were carried out at 4 \(^\circ\) C.

2.2.3. 5a-reductase assay

In order to determine the anti-5a-reductase activity of the steroids in human prostatic microsomes, it was used a method based on a dispersive liquid–liquid microextraction (DLLME) procedure, followed by the GC–MS method previously described [19]. The synthesized steroids were dissolved in DMSO and diluted in 40 mM sodium phosphate buffer (pH 6.5).

As a screening assay, to determine the percentage of reductase inhibition of each compound, it was used 50 \(\mu\)g of prostate microsomal protein, 500 nM of T, 1 mM of DTT, 25 \(\mu\)L of DMSO and 1 \(\mu\)M of each steroidal compound in 40 mM of sodium phosphate buffer (pH 6.5), in a final reaction volume of 0.5 mL. To determine the IC\(_{50}\) value of the steroids that presented an anti-reductase activity higher than 60%, it was used different concentrations of each inhibitor (0.01–2 \(\mu\)M). The enzymatic reaction was initiated by the addition of 250 \(\mu\)L of reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Sigma–Aldrich) and incubations were performed in a shaking water bath at \(37^\circ\) C for 60 min. The reductase reactions were stopped by the addition of 500 \(\mu\)L of MeCN in ice and the steroids, T and DHT, were extracted by DLLME. Then, to this enzymatic mixture it was added 50 \(\mu\)L of Td\(_3\) (175 nM) and DHT-\(^{13}C_3\) (175 nM) and 50 \(\mu\)L of the high purity extractive solvent trichloroethylene (C\(_2\)HCl\(_3\)) (Fluka). This mixture was transferred to a conical tube with 3 mL of H\(_2\)O, the tubes were hand-shaken and centrifuged at 5000 \(\times\) g for 3 min. After centrifugation, the lower phase (30 \(\mu\)L) that contained the extracted steroids was transferred to a new vial and dried using a gentle nitrogen stream at room temperature. After evaporation, the steroids were silylated by addition of 30 \(\mu\)L of a solution containing N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, 98.5% purity grade) (Fluka), ammonium iodide (NH\(_4\)I, 99% purity grade) and 1,4-dithioerythritol (DTE, 99% purity grade) (Sigma–Aldrich) (MSTFA + NH\(_4\)I + DTE) during 5 min in a household microwave (600 W), and 1 \(\mu\)L of the extract was injected in the GC–MS system. The GC–MS conditions are according our previously reported method [19]. All the experiments were performed in triplicate. As a reference control it was used the R1 finasteride at 1 \(\mu\)M (Sequoya Research Products Ltd, Pangbourne, UK).

2.3. Anti-proliferative effects

2.3.1. Cell culture

The androgen-sensitive human prostate adenocarcinoma cells, LNCaP, were purchased from the American Type Culture Collection
(ATCC, Manassas, VA, USA) and maintained in RPMI 1640 medium (ATCC modification) with 4.5 g/L d-glucose, 2.383 g/L HEPES buffer, 300 mg/L L-glutamine, 1.5 g/L sodium bicarbonate, 110 mg/L of sodium pyruvate, 1% penicillin-streptomycin-amphotericin B and 10% of heat-inactivated fetal bovine serum (FBS) (Gibco Invitrogen Co., Paisley, Scotland, UK). The LNCaP cells were regularly grown at 37 °C in 5% CO₂ atmosphere and medium was changed every three days. For LNCaP assays, cells were used between the passages 5 and 20, since beyond 25 passages reduced androgen responsiveness was already observed [24,25].

The biological effects of the steroidal compounds that presented an anti-reductase activity higher than 60% were evaluated in LNCaP cells. Before the addition of compounds, cells were cultured during 3 days in a steroid-free RPMI medium with L-glutamine but without phenol red (Gibco Invitrogen Co), containing 1 mmol/L of sodium pyruvate, 1% of penicillin-streptomycin-amphotericin B and 5% of pre-treated charcoal heat-inactivated fetal bovine serum (CFBS), in order to avoid the interference of the steroids present in FBS and of the oestrogenic effects of phenol-red [26]. After that, steroids in study were added with or without the proliferation inducing agent (T or DHT). All the experiments were performed according to these conditions and the medium and drugs were refreshed every 3 days.

2.3.2. Cell viability

To evaluate the effects of each steroidal compound in LNCaP cells viability, the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) (Sigma–Aldrich, Saint Louis, USA) and lactate dehydrogenase (LDH) release (Promega Corporation, Madison, USA) assays were performed. After confluence, LNCaP cells were cultured in 96-well plates at a cellular density of 3 × 10⁴ cells/mL (for 2, 3 and 4 days) and 1.5 × 10⁴ cells/mL (for 6 days) and after 3 days of steroid-free culture the cells were treated with different concentrations of each compound (1–50 μM) without any proliferation inducing agent, or with 10 nM of T, or with 10 nM of DHT during 2, 3, 4 and 6 days. Cells incubated with 10 nM of the proliferation inducing agent plus 0.05% of DMSO were used as control. Finasteride was used as a reference RI.

After each incubation time, MTT (0.5 mg/mL) was added to each well and cells were incubated for 2 h and 30 min at 37 °C in 5% CO₂. The formazan was quantified spectrophotometrically by addition of DMSO:isopropanol mixture (3:1). The LDH release was measured by using CytoTox 96 nonradioactive cytotoxicity assay kit, according to the manufacturer’s protocol. The results are expressed as a percentage of the untreated control cells. All the assays were performed in triplicate in three independent experiments.

2.4. Statistical analysis

Statistical analysis of data was performed using analysis of variance (ANOVA) followed by Bonferroni test for multiple comparisons and values of p < 0.05 were considered as statistically significant. The data are expressed as the mean ± SEM.

3. Results

3.1. Chemistry

The synthesis of compound 2 (Scheme 1), the C-3 deoxygenated derivative of 4-androsten-3-one-17β-carboxylic acid 1, was performed by submitting 1 to a reduction with sodium borohydride in trifluoroacetic acid, glacial acetic acid, and acetonitrile giving compound 2, in 71% yield [27].

The amides 3, 4 and 5 (Scheme 1) were obtained in 69%, 54% and 35% yield, respectively, by reacting acid 1 with tert-butylamine, isopropylamine or n-hexylamine, in dimethylformamide and dichloromethane, using triethylamine and the coupling agent BOP [28] probably through the intermediate 7. In spite of amide 3 has already been prepared in 89% [20] and in 95% [29] using thionyl chloride followed by tert-butylamine, in this work we present a one-step reaction using milder conditions.

An attempt to prepare amide 6 from 1 (Scheme 1) using the abovementioned conditions with the amine 2,5-bis-(trifluoromethyl)aniline, afforded 7 in 73% yield. In this case, the activated intermediate ester 7 was unable to react with the amine 2,5-bis-(trifluoromethyl)aniline to give 6, probably due to the weak nucleophilicity of the deactivated aromatic amine 2,5-bis-(trifluoromethyl)aniline. Consequently, the final product was only compound 7. In order to overcome this limitation, amide 6 was obtained by a two-step reaction sequence [30]. In the first step, the C-17 carboxylic acid 1 was activated to the respective acyl chloride by treatment with thionyl chloride, in dry tetrahydrofuran and in the presence of dry pyridine. In the second step, the acyl chloride was subjected to reaction with 2,5-bis-(trifluoromethyl)aniline in dry tetrahydrofuran and in the presence of dry pyridine providing compound 6 in 35% yield.

3.2. 5α-Reductase activity

To evaluate the anti-5α-reductase activity of each steroidal compound in human prostatic microsomes, it was applied a DLLME-GC–MS method previously developed by our group, that identifies and quantifies T and DHT, using the internal standards Td₃ and DHT-¹⁴C₃ [19]. The ratio between T/Td₃ and DHT/DHT-¹⁴C₃ allowed the quantification of T and DHT produced after 5α-reductase reaction estimating the anti-5α-reductase activity of each compound.

Firstly, it was evaluated the percentage of 5α-reductase inhibition (%) of the synthesized RIs at 1 μM (Table 1 and Fig. 3A). As a reference compound it was used the RI finasteride, that presented an 84.62 ± 1.21% of 5α-reductase inhibition, which is in accordance to what has been already described (80%–90%) in human prostate microsomes [31]. Three of the studied steroids, 3, 4 and 5, presented an anti-reductase activity higher than 60%, 73.08 ± 3.05%, 69.33 ± 1.03% and 63.56 ± 1.08%, respectively. For these steroids, the IC₅₀ was also determined (0.37 μM, 0.46 μM and 0.61 μM, respectively) (Table 1 and Fig. 3B). Our results demonstrated that steroid 3 is the most potent RI in human prostate microsomes.

3.3. Effects of steroids in LNCaP cells viability

The effects of the most potent RIs 3, 4, 5 and finasteride (1–50 μM) in LNCaP cells viability were investigated using MTT and LDH release assays after 2, 3, 4 and 6 days of treatment.

To evaluate the adequate concentrations of the inducing proliferation agents, LNCaP cells were cultured with different concentrations of T or DHT (0.1–100 nM). In our experimental conditions, the concentration of 10 nM for T and DHT was selected as the most adequate to induce proliferation of LNCaP cells in vitro (data not shown).

It was evaluated the biological effect of RIs 3, 4, 5 and finasteride (1–50 μM) on LNCaP T-treated cells and, as observed in Fig. 4, RIs induced a decrease in cell viability in a dose- and time-dependent manner. Compound 5 and finasteride caused a significant (p < 0.05; p < 0.01; p < 0.001) decrease in cell viability for all the concentrations and all days of treatment, except for the lowest concentration (1 μM) after 2 days. Compounds 3 and 4 presented a significant (p < 0.05; p < 0.01; p < 0.001) reduction in cell viability for the higher concentrations (10–50 μM) and all times of incubation, except after 2 (1 and 5 μM) and 3 (1 μM) days of treatment.
for the lower concentrations. As shown in Fig. 4E and F, steroid 3 is the most efficient and finasteride the less potent in decreasing cell viability. Cells treated with T were considered as control.

It was also evaluated the LDH release in T-treated LNCaP cells after 3 days of treatment with compounds. As finasteride, steroids 3 and 5, for the higher concentration (50 μM), induced a significant (p < 0.05; p < 0.01) release of LDH (Fig. 5), therefore the effects of these compounds were further studied between 1 and 25 μM. In addition, to confirm if compounds caused by themselves any effect in LNCaP cell growth, RIs treated-cells were cultured in the absence of T or DHT for 3 days. As it can be observed in Fig. 6, all the studied steroids showed a minimal growth-inhibitory activity. Only for the higher concentration (25 μM), steroids 3 and 5 presented a significant (p < 0.05) reduction in cell viability.

The ability of compounds to inhibit the growth of DHT-stimulated LNCaP cells was also evaluated after 3 and 6 days. Our results demonstrate that RIs present a significant (p < 0.05; p < 0.01) inhibitory growth of DHT-treated cells (Fig. 7). Though, to understand if these effects are dependent on reductase inhibition, it was compared their effects on T versus DHT-treated cells. By the analysis of Fig. 7A and B, it is observed that inhibition, it was compared their effects on T versus DHT-treated cells. By the analysis of Fig. 7A and B, it is observed that in cell viability, after 3 days and for all the concentrations after 6 days. Steroid 4 caused significant differences (p < 0.05; p < 0.01; p < 0.001) for all the concentrations and all days of treatment, except for 1 μM after 3 days (Fig. 7C). Finally, for steroid 5 it was only observed significant differences (p < 0.05; p < 0.01) for the higher concentrations (10 and 25 μM) after 6 days (Fig. 7D), which suggests that the effects of this steroid on DHT-treated cells is only time-dependent.

### Table 1

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Reductase inhibition (%) ± SEM</th>
<th>IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29.55 ± 3.09</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>11.04 ± 3.42</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>73.08 ± 3.05</td>
<td>0.37</td>
</tr>
<tr>
<td>4</td>
<td>69.33 ± 1.03</td>
<td>0.46</td>
</tr>
<tr>
<td>5</td>
<td>63.56 ± 1.08</td>
<td>0.61</td>
</tr>
<tr>
<td>6</td>
<td>22.44 ± 2.03</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>49.20 ± 2.51</td>
<td>–</td>
</tr>
<tr>
<td>Finasteride</td>
<td>84.62 ± 1.21</td>
<td>0.096</td>
</tr>
</tbody>
</table>

### Discussion

In this study it was investigated the inhibitory activity of 5α-R of a set of synthesized steroids. It is known that one of the key structural requirements for 5α-R inhibition is the presence of a 4-en-3-one function in the A-ring and a lipophilic 17β-side chain in the D-ring of steroids [6]. Furthermore, it has been found that the presence at C-17β of lipophilic amides or esters enhances potency by binding to a lipophilic pocket on the enzyme [6]. In this study, these features were investigated by studying different C-17β chemical modifications (amide, ester or carboxylic acid functions) in the potential steroidal RIs.

The evaluation of anti-5α-R activity of each steroid in human prostatic microsomes was performed by a DLLME-GC–MS previously developed method [19]. Based on the results displayed in Table 1, it is possible to infer that C-17β carboxylic acid analogues (1 and 2) of T are weak inhibitors of 5α-R, particularly the 3-deoxo analogue 2. In fact, several descriptions referred the importance of a sp3-hybridized center at C-3 and C-4 positions of steroids for the 5α-R inhibitory activity [6]. Therefore, the lack of that center at C-3 as in 2, results in a decrease in the activity (11.04 ± 3.42% inhibition). Concerning analogue 1, it was already reported as a competitive inhibitor of 5α-R, with 87.7% inhibition for the microsomal enzyme of human skin [6,32]. However, in our experimental conditions, using the prostate microsomal enzyme, analogue 1 showed only 29.55 ± 3.09% inhibition.

Among the amides synthesized and evaluated, the N-tert-butylicarboxamide 3 (Scheme 1) showed the best inhibitory activity, with an IC50 of 0.37 µM (Table 1). In this case, we combined in the same molecule, the A-ring of the substrate T and the C-17β carboxamide of finasteride (Fig. 2), which resulted in a potent inhibitor.

Regarding the N-propylcarboxamide 4 and the N-hexylcarboxamide 5 (Scheme 1) it is possible to infer that these steroids are also strong inhibitors (IC50 of 0.46 and 0.61 µM, respectively) (Table 1). Further, the N-propylcarboxamide 4 is slightly more active than the N-hexylcarboxamide 5, however both of them are less active than N-tert-butylcarboxamide 3, showing that a hindered N-alkyl group, as in 3, or a shorter N-alkyl linear chain, as in 4, in the C-17β carboxamide function favours the 5α-R inhibitory activity.

Concerning compound 6, in spite of having in the C-17β position the same group as dutasteride, the N-[2,5-bis(trifluoromethyl) phenyl]carboxamide, surprisingly it only exhibits minimal inhibitory activity (22.44 ± 2.03%). This result reveals that along with the C-17β carboxamide group, the steroidal A-ring type also determines the
Effects of RIs, steroids and finasteride on cell viability. LNCaP cells were cultured with different concentrations of each RI (1–50 μM) during 3 days. Cells cultured without any proliferating agent (DHT or 17β-estradiol) were used as controls. Results are the mean ± SEM of three independent experiments, performed in triplicate. Significant differences between the control and cells with each RI are denoted by * (p < 0.05), ** (p < 0.01) and *** (p < 0.001). Finasteride (F) was used as reference RI.

Fig. 4. Effects of RIs (F), testosterone (T), finasteride (F) and nasteride (F) in cell viability of T-treated LNCaP cells, evaluated by MTT assay. Comparison of the effects of all the RIs, after 3 (E) and 6 (F) days of treatment. LNCaP cells were cultured with different concentrations of each RI (1–50 μM) and T at 10 nM during 2, 3, 4 and 6 days. Cells cultured with T represent the maximum of cell viability and were considered as control. Results are the mean ± SEM of three independent experiments, performed in triplicate. Significant differences between the control and cells with each RI are denoted by * (p < 0.05), ** (p < 0.01) and *** (p < 0.001). Finasteride (F) was used as reference RI.

Potency of inhibitors. Regarding ester 7, our results demonstrate that it is a moderate inhibitor (49.20 ± 2.51% inhibition).

In this study it was also evaluated the effect of the most potent RIs, steroids 3, 4, 5 and finasteride in LNCaP cells viability. This cell line is a human androgen-responsive prostate cancer cell line, that is a good model to study hormonal therapies for this disease [33]. LNCaP cells are a biological model similar to a non-invasive and low-grade tumour, being differentiated prostate features...
This cell line responds positively to growth stimulation induced by oestrogens or androgens in vitro and in vivo. Therefore, the effects of the most potent steroids were studied in LNCaP cells treated with 10 nM of T or DHT.

The results obtained with T-treated LNCaP cells with RIs finasteride (F), 3, 4, 5 and finasteride after 2, 3, 4 and 6 days indicate that compounds induced a significant decrease in cell viability, being steroid 3 the most potent, even more than finasteride. Other studies also demonstrated that finasteride inhibited growth of LNCaP cells treated with T, though only after 9 days of treatment. Golbano J. M. et al. (2008) demonstrated that finasteride significantly induced a reduction in LNCaP cell viability in a dose-
and time-dependent manner due to apoptosis via Bcl-2 family and caspase activity [33]. To investigate if the biological effects induced by all the RIs were due to 5α-R inhibition, it was compared their effects in T- versus DHT-treated cells. It was observed significant differences for all steroids, being the effects on T-treated cells more remarkable than on DHT-treated cells. For steroid 5 this effect was only noticeable with the extension of treatment. These results suggest that these steroids induced the reduction in cell viability in a 5α-R-dependent manner, by inhibiting 5α-reductase and the conversion of T into DHT. Nevertheless, it cannot be discarded the hypothesis that other mechanisms may also be involved, since in the presence of DHT, these RIs also induced a slight decrease in LNCaP cell viability, especially with the extension of treatment. Other studies had already demonstrated that finasteride reduce cell growth of DHT-treated LNCaP cells in a dose-dependent manner [37–41].

In order to understand if the ability of steroids to decrease viability of T- and DHT-treated cells was not dependent on their intrinsic toxicity, it was also evaluated their effects in cells without a stimulatory effect. Our data showed that all the steroids induced a minimal growth-inhibitory activity in cells, on their own. This observation was already reported for finasteride in other studies [33,37–40].

5. Conclusion
Taking into account the studied compounds, it is possible to conclude that the C-17β lipophilic carboxamides along with the 3-keto-Δ4 moiety in the A-ring seem to be essential key features for achieving 5α-reductase inhibitory activity, being the steroid with a C-17 N-ter-butylocarboxamide group (3), the best RI. Furthermore, steroids 3, 4, and 5 as well as finasteride induced a decrease in viability of stimulated LNCaP cells in a 5α-R-dependent manner, being the synthesized steroids 3, 4, and 5 even more effective than finasteride. Our results also suggest that other mechanisms are involved in diminishing cell viability. This study can help in the future design of new steroidal RIs contributing to the discovery of new drugs with fewer side effects.

Conflict of interest
The authors have no conflict of interest to declare.

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